REDOX MODULATION OF GLUCOSE-6-P DEHYDROGENASE IN *ANACYSTIS NIDULANS* AND ITS 'UNCOUPLING' BY PHAGE INFECTION

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1. Introduction

We have reported [1] that phage infection induces drastic changes in the respiratory carbon metabolism of *Anacystis nidulans*, a photoautotrophic cyanobacterium:

(i) Glucose-6-P dehydrogenase (G6PDH), the first enzyme of the oxidative hexose monophosphate (HMP) pathway, undergoes a transition into a hyperactive form;

(ii) Simultaneously with the activation of G6PDH, the carbon flow via the HMP shunt increases.

In the heterotrophic prokaryote *Escherichia coli* phage infection has been reported to decrease [2,3] or leave unaltered [4] the activity of the HMP shunt.

We report here that the different responses of the autotrophic and heterotrophic prokaryotes to phage infection can be explained by the different regulatory properties of their G6PDHs. In the healthy cyanobacterial cell, G6PDH is maintained in a low activity form by a powerful reducing system. Phage infection interferes with the operation of this reducing system, leading to an oxidative transformation of the enzyme into a hyperactive form. No similar regulatory mechanism is known to work in *E. coli* and other heterotrophic bacteria [5].

2. Materials and methods

Anacystis nidulans was grown and infected by cyanophage AS-1 [6] as in [7]. Under the conditions used, the lytic cycle lasted for 6–8 h [8,9]. Phage adsorption was completed in 20 min [10]. At 3 h after infection the cells were harvested by centrifugation, resuspended in a 3-fold vol. of ice-cold, 50 mM Tris–HCl buffer (pH 7.5) and sonicated under cooling. If not stated otherwise, 10 000 × *g* supernatants were used in the assays. For further details, see [1] and the legends to the figures. The amount of NADP + NADPH was determined according to [11].

3. Results

3.1. *G6PDH is under redox control*

The G6PDH activity in freshly prepared 10 000 × *g* (membrane containing) supernatants from sonicates of *Anacystis* cells rapidly increased (4–5-times) upon aeration (table 1). The system was extremely sensitive to oxygen. In open vessels the G6PDH activity of the supernatants steadily increased for several days, even at 4°C.

Treatment of the fresh supernatant with oxidants, like oxidized glutathione (GSSG), dehydroascorbate (DHA) or H_2O_2, also increased the G6PDH activity (table 1). This effect proved to be reversible. G6PDH activated by oxidation was deactivated in 10–20 min if the system was flushed with argon (table 1). The results suggest the presence in the extracts of stored reducing power which competes with oxidants in the modulation of G6PDH activity. Light, probably the ultimate source of the reducing power, was not necessary for the reductive enzyme modulation, although the G6PDH activity of the oxidized supernatant decreased more rapidly, and to a somewhat greater extent, if the sample flushed with argon was illuminated as well (table 1). The effect of illumination could be attributed to the operation of an active photosynthetic electron-transport chain because:

(i) The light effect was reduced in the presence of 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea

Table 1
Modulation of G6PDH in 10 000 × *g* supernatants from sonicates of *Anacystis nidulans*

Samples used for pre-treatment	Pre-treatment (pre-incubation) before enzyme assay for 20 min[a]	G6PDH activity[b]	
		Healthy	Infected
Fresh	None	1.2	7.3
Fresh	Aeration	6.3	7.6
Fresh	GSSG (20 mM)	6.1	7.7
Fresh	DHAA (20 mM)	5.9	7.5
Fresh	H_2O_2 (0.1%)	6.0	7.7
Aerated	Flushed with argon in the light[c]	1.4	7.4
Aerated	Flushed with argon in the light + DCMU (10 μM)	3.9	–
Aerated	Flushed with argon in the dark	3.5	–
Aerated	DTT (5 mM)	2.2	3.0
Aerated	NADPH (50 μM)	–	7.5
Aerated	NADPH (50 μM) flushed with argon in the light	–	1.9
Aerated	NADP (50 μM)	–	7.6
Aerated	NADP (50 μM) flushed with argon in the light	–	1.9
Aerated	NADP (50 μM) flushed with argon in the dark	–	4.1

[a] Aliquots of supernatants were pre-treated as shown in the table and 5 μl of the pre-treated supernatant was injected into the assay system (1 ml)
[b] μmoles of NADP reduced during the first 1 min of the reaction per 10^8 cells. The assay system contained 2 mM G6P and 0.12 μM NADP
[c] 2.5 mW/cm²

(DCMU), an inhibitor of the photosynthetic electron-transport chain (table 1);

(ii) No light effect was obtained in the 100 000 × *g* supernatants which are devoid of photosynthetic membrane fragments (not shown).

The effect of reducing compounds like reduced glutathione (GSH) and ascorbic acid (AA) on G6PDH activity was erratic. The powerful, non-physiological reductant dithiothreitol (DTT), however, decreased the G6PDH activity of aerated samples (table 1).

We conclude that the *Anacystis* G6PDH is under redox control. The question then arises as to whether the changes in G6PDH activity, due to redox modulation, can be explained in terms of properties of the enzyme already studied in some detail.

3.2. *The kinetics and apparent molecular weight of G6PDH are affected by oxidation and reduction*

Different kinetic forms of the cyanobacterial G6PDH have been described [12]. The 'hyperactive' form exhibits a burst, the 'hypoactive' form shows a lag, during enzyme assay. The two forms are in equilibrium [12], although the equilibrium can be shifted. The G6PDH in fresh *Anacystis* extracts is present predominantly in the hypoactive form [1].

Our experiments showed that treatment of the fresh supernatant with oxidants results in a shift in the equilibrium toward the hyperactive form. Flushing of the oxidized sample with argon reconverted the enzyme into its hypoactive state. In the oxidized sample, the burst was slight and the steady state value of enzyme activity (V_∞) was higher than in the reduced sample (fig.1).

Conditions known to lead to the formation of highly active cyanobacterial G6PDH form(s) (e.g., high enzyme or G6P concentration, slightly acidic pH) induce enzyme aggregation [1,12].

We found that oxidation also leads to an increase in the app. M_r of the enzyme, suggesting enzyme aggregation (fig.2).

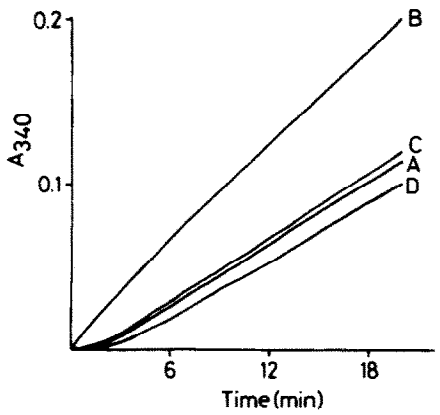

Fig.1. The effect of oxidizing and reducing conditions on the kinetics of NADP reduction by cyanobacterial G6PDH. Direct recording of enzyme activity. (A) Freshly prepared 10 000 × *g* supernatant; (B) fresh supernatant aerated for 20 min before assay; (C) aerated supernatant (B) kept under argon for 30 min before assay; (D) 5 μl aerated supernatant (B) diluted in 0.8 ml 50 mM Tris–HCl buffer (pH 7.5) and incubated for 10 min before assay. The reaction was initiated by adding 5 μl supernatant to the complete reaction mixture (10 mM G6P, 0.5 mM NADP in 50 mM Tris–HCl buffer (pH 7.5)) except for (D) in which the reaction was started by the addition of the substrates.

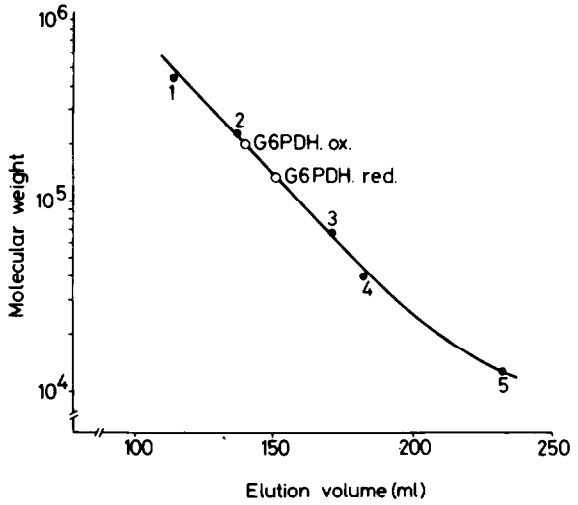

Fig.2. The effect of oxidizing and reducing conditions on the app. M_r of the G6PDH of *Anacystis*. Aliquots (2 ml) of freshly prepared, 10 000 × *g* supernatant were oxidized by aeration for 20 min or reduced by treatment with 5 mM DTT for 20 min. The samples were chromatographed on 2 × 80 cm Sephadex G-200 columns. The oxidized sample was eluted by 0.1 M Tris–HCl buffer (pH 7.5). The reduced sample was eluted by the same buffer containing 5 mM DTT and 10 mM β-mercaptoethanol. The M_r standards were: 1 = ferritin (440 000); 2 = catalase (230 000); 3 = bovine serum albumin (68 000); 4 = horse radish peroxidase (40 000); 5 = cytochrome *c* (12 000); (all Sigma).

The aggregated state of the oxidized enzyme is also reflected in its kinetic properties. The aggregated form of the cyanobacterial G6PDH, induced by low pH and/or high substrate concentration, is known to obey regular Michaelis-Menten kinetics, whilst the deaggregated form shows a strong cooperative effect of G6P [12]. We found that the oxidized form displays normal Michaelis-Menten kinetics, whereas the reduced form exhibits cooperativity when V_i is plotted against G6P concentration (not shown).

3.3. *Interaction between the redox modulation of G6PDH and other factors modulating the enzyme activity*

We found a complex interaction between the effects of redox modulators and those of other factors known to affect the activity of the enzyme. Of the data obtained, only those showing the effect of dilution on the oxidized enzyme are presented.

The oxidized, slightly hyperactive enzyme was transformed into hypoactive form upon dilution (fig.1). We therefore conclude that the equilibrium of the hypoactive ⇌ hyperactive states of the *Anacystis* G6PDH is under redox control. This control, however, is affected by other controlling factors, e.g., enzyme and substrate concentration [1,12].

3.4. *Virus infection uncouples the redox modulation of G6PDH*

The question arises which of the factors able to modulate the *Anacystis* G6PDH is actually responsible for the phage-induced hypoactive to hyperactive shift in G6PDH forms. If this shift were due primarily to oxidative processes, the G6PDH extracted from infected cells should not be subjected to oxidative modulation. As shown in table 1, oxidants indeed had no significant effect on the activity of G6PDH in extracts from phage-infected cells, suggesting that the enzyme is in an oxidized state. Surprisingly, the enzyme activity did not decrease under reducing conditions (flushing with argon) (table 1). To explain the apparent discrepancy, 3 possibilities were considered:

(i) The reaction leading to the formation of the high activity form of G6PDH in the infected cell is not oxidative;
(ii) The reaction is oxidative but, unlike the process operating in the healthy cells, it is irreversible;
(iii) A component which is responsible for the reducibility of G6PDH in the extracts from the control cells is missing from the extracts of infected cells.

We have found that alternative (iii) is correct. Upon addition of either NADPH or NADP to the 10 000 × *g* supernatants from infected cells, the G6PDH activity rapidly declined under anaerobic conditions even in the dark. Illumination speeded up the process but was not necessary. Addition of NADPH or NADP to the extracts in the presence of air was, as expected, ineffective (table 1).

The fact that NADP was just as effective as NADPH in restoring the reductive modulatability of the enzyme showed that the strong reducing power of the system was not lost upon infection. The results suggested that rather the NADP + NADPH level may be too low in the infected cells. Indeed, we found 11.6 and 6.0 nmol/ml of NADP + NADPH in the extracts from healthy and infected cells, respectively. We conclude, therefore, that in the infected cells the amount of NADP + NADPH is not enough to yield sufficient NADPH necessary to couple the reducing power of the cell with the reduction of G6PDH.

4. Discussion

The reducing system regulating enzyme activity in *Anacystis* has a new feature when compared to those operating in chloroplasts (reviews [13,14]); it is capable of decreasing the activity of oxidized G6PDH under anaerobic conditions even in the dark. The *Anacystis* system is almost saturated with stored reducing power, which becomes available for the reductive modulation of G6PDH as soon as the competition by O_2 has been abolished.

In *Anacystis*, the endogenous reducing power is coupled with the reductive modulation of G6PDH via the NADP/NADPH redox pair. The mediator of H-transfer between NADPH and G6PDH is not known but it may well be thioredoxin. This is likely because treatment with 5 mM DTT led to a dramatic decrease in G6PDH activity in the 10 000 × *g* supernatants, even in the air. It is known that this concentration of DTT can reduce the endogenous thioredoxin(s) and modulate enzymes via thioredoxin-H_2 but it is generally unable to modulate the same enzymes directly [15]. Therefore, we propose the operation in *Anacystis* of an NADPH-linked thioredoxin reductase, in contrast to the ferredoxin-dependent thioredoxin reductase known to be involved in the reductive modulation of chloroplast enzymes [14]. NADP-dependent thioredoxin reductases are known to be present in prokaryotes [16].

A most important factor inducing the metabolic changes in infected *Anacystis* is the decrease in the amount of NADP + NADPH. The reason for this decrease is not known. One possibility is the temporary leakage of small M_r compounds from the phage-infected cells, as described for *E. coli* [17]. If this is the case, the effect of leakage must be long lasting because the infected cells remain short of NADP + NADPH even in the later stages of the lytic cycle.

Clearly, NADPH plays a crucial role in the metabolic regulation in *Anacystis*. A variety of virus-induced processes are competing for the NADPH pool of the cells. It is logical to assume that those involved in viral DNA synthesis must have a priority. An increased consumption of NADPH (due to intensive viral DNA synthesis), an increase in NADPH-oxidase activity [1], a decrease in the level of glutathione reductase (not shown) and finally a reduced NADP + NADPH level, seem to be responsible for a more oxidative milieu in the infected cell. This leads to the formation of the oxidized, hyperactive form of G6PDH which, in a feed-back type of process, produces more NADPH in an 'effort' to meet the increased NADPH requirements created by phage infection. The whole system represents a novel type of metabolic control characteristic for the phage-infected autotrophic cell.

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